Interleukin-6 Promoter Polymorphism Modulates the Effects of Heavy Alcohol Consumption on Early Carotid Artery Atherosclerosis

The Carotid Atherosclerosis Progression Study (CAPS)

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Background and Purpose—A J-shaped relationship has been demonstrated between alcohol and both clinical cardiovascular events and carotid atherosclerosis. A similar J-shaped relationship has been found between alcohol intake and inflammatory markers. If inflammation were on the intermediate causal pathway between alcohol intake and atherosclerosis, then genetic determinants of enhanced inflammation would be expected to modify this relationship.

Methods—In a large community population (n=1000; age, 50 to 65 years), we studied the effects of the interleukin-6 (IL-6)-174 polymorphism and gene-alcohol interactions on common carotid artery intima-media thickness (CCA-IMT) and carotid plaque.

Results—The CC genotype was associated with significantly higher IL-6 levels; the odds ratio (OR) for IL-6 in the top quartile was 2.07 (95% CI, 1.16 to 3.72; P<0.014). Interactions were seen between genotype and alcohol consumption for both IL-6 levels and CCA-IMT. In individuals who drank >30 g/d of alcohol, the CC genotype was associated with higher IL-6 levels, elevated CCA-IMT (P=0.001), and increased risk of carotid plaque (OR, 3.64; 95% CI, 1.15 to 11.54; P=0.028). The J-shaped relationship between alcohol intake and IMT was seen only for the CC genotype.

Conclusions—These data suggest that the IL-6-174 promotor polymorphism may modulate the effects of alcohol on carotid atherosclerosis. These data support the hypothesis that inflammation forms part of the intermediate causal pathway between alcohol intake and atherosclerosis. (Stroke. 2003;34:402-407.)

Key Words: alcohol ■ atherosclerosis ■ genes ■ inflammation ■ interleukins

Carotid artery stenosis accounts for a significant proportion of ischemic stroke. There is evidence for a strong genetic component to carotid atherosclerosis, with genes accounting for as much as one third of the overall risk. Strong correlations have been found between inflammatory markers and measures of carotid atherosclerosis, although these correlations appear to be greatly influenced by conventional vascular risk factors. Genetic determinants of inflammation may therefore be important in the pathogenesis of atherosclerosis, particularly in individuals exposed to environmental proinflammatory stimuli.

Large-scale epidemiological studies have consistently shown that nondrinkers and heavy drinkers have increased vascular mortality and stroke risk compared with light to moderate drinkers (<2 U/d). The reasons for this J-shaped relationship are incompletely understood, but suggested mechanisms include alterations in blood lipids, coagulation profile, insulin resistance, and altered inflammatory responses. A similar J-shaped relationship has been demonstrated between alcohol intake and carotid atherosclerosis, suggesting that this relationship between alcohol and clinical vascular risk may be mediated by the effect of alcohol on atherosclerosis risk. A similar J-shaped relationship has been found between alcohol intake and inflammatory markers, and inflammation and cytokine responses may provide a key mechanism by which alcohol mediates atherosclerosis risk.

There is growing evidence that the proinflammatory cytokine interleukin-6 (IL-6) is an active participant in the process of atherogenesis. A genetic variant in the IL-6 gene (IL-6-174 G/C) has been associated with quantitative changes in IL-6. Recent studies have found associations between the C allele of this polymorphism and cardiovascular disease. Moderate alcohol intake has been shown to alter activation of transcription factors involved in IL-6 production, and the
IL-6-174 polymorphism is located close to binding areas for these transcription factors. If inflammation were on the intermediate causal pathway between alcohol intake and atherosclerosis, then genetic determinants of enhanced inflammation would be expected to modify this relationship.

In a community-based population, we studied the impact of the IL-6-174 polymorphism and the effects of gene-alcohol interactions on common carotid artery intima-media thickness (CCA-IMT) as a measure of early atherosclerosis. Furthermore, we performed a case-control study to determine whether the IL-6-174 genotype is a risk factor for the presence of established internal carotid artery plaque as a marker of established atherosclerosis.

Subjects and Methods

Study Population
The study sample was drawn from participants in the Carotid Atherosclerosis Progression Study (CAPS), details of which have been published elsewhere. All members of 2 German health insurance companies ≥40 years of age (n = 15 879) who lived within a radius of 50 km from 5 study sites in Western Germany were invited to participate. Within a predefined time limit, 5460 agreed to participate. For this study, we identified 2 subgroups from this overall population to determine whether the IL-6 genotype was associated with early carotid atherosclerosis as indicated by increased IMT and with established carotid plaque as a marker of more advanced atherosclerosis. To answer the first question, we studied the first 1000 consecutively recruited subjects in the age range of 50 to 65 years from the total sample of 5460. In this study, IMT values were correlated with genotype and other cardiovascular risk factors. This population size was chosen to enable the detection of a difference in IMT between genotypes of 0.05 mm with P = 0.01 and a power of 0.9. In the second study, we identified all cases from the entire cohort of 5460 with carotid plaque present and DNA available (n = 194, 62% male; age range, 36 to 85 years). We matched these with plaque-free controls (n = 523) from the same cohort. Matching was performed for age (by 10-year categories), sex, history of arterial hypertension, diabetes, and coronary heart disease, cholesterol, body mass index (BMI), smoking category, and socioeconomic status but not for alcohol consumption. One hundred eighty-one subjects were common to both the IMT and carotid plaque studies.

Vascular risk factors were assessed by use of a standardized computer-assisted interview technique and examination by a physician trained in vascular medicine. Risk factors determined included the following: pack-years of smoking, current smoking status, BMI, hypertension, diabetes, and coronary heart disease, cholesterol, body mass index (BMI), smoking category, and socioeconomic status but not for alcohol consumption. One hundred eighty-one subjects were common to both the IMT and carotid plaque studies.

Ultrasound Imaging
For ultrasonic examinations, a 7.5- to 10.0-MHz linear-array transducer was used (P700SE, Phillips Medical System). Preprocessing configurations were held constant during all examinations. The gain was adjusted so that the least dense arterial wall interface was just visible. Using antero-oblique insonation, we visualized far-wall CCA-IMT within the CCA. Images were digitally captured during the systole of a single heartbeat for offline measurements. Vertical and horizontal calibration measurements were performed every 100th measurement with an ultrasound assurance phantom.

The method used for IMT measurements, including the analysis of images with semiautomatic image analysis software, has been described in detail previously. High levels of interobserver and intraobserver reproducibility were obtained. The mean length of the arterial segment in which IMT was determined was 14.35 mm for the left CCA-IMT, 12.85 mm for the right CCA-IMT, and 3.45 mm for the ICA-IMT on both sides.

Carotid plaque in the distal CCA or proximal internal carotid artery was defined as any obscuration of the free luminal vessel surface with a distance between the luminal interface and the medial-adventitial interface >1.7 mm. Interobserver reliability for 4 different observers for 30 carotid plaques gave a linear regression of r = 0.76 to 0.90 and ± 2 SD of the mean of the difference between observers of 5% to 10%.

Laboratory Methods
DNA was extracted from whole blood with Nucleon kits (Tepnel Life Sciences). The IL-6-174 polymorphism was genotyped by use of the polymerase chain reaction and restriction enzyme digestion with NolIII, producing products of 198 base pairs for the g allele and 140 and 58 base pairs for the c allele. Products were visualized on agarose gel stained with ethidium bromide. Genotypes were confirmed by sequencing. Polymerase chain reaction conditions and primers were according to the methods of Fishman et al.

In the first consecutive 500 subjects enrolled in the IMT study, fasting serum stored at −70 °C was defrosted once only for determination of high-sensitivity IL-6 levels with a commercially available enzyme-linked immunosorbent assay (R&D Systems; sensitivity, 0.094 pg/mL; range, 0.156 to 10 pg/mL).

Statistical Methods
Data were analyzed with SPSS (version 10). CCA-IMT was analyzed as a continuous variable with multiple linear regression. The mean of the right and left CCA-IMT values was skewed; therefore, the reciprocal IMT was used to normalize the distribution. Geometric mean IMT values are given in the text for clarity. Age, BMI, mean systolic and diastolic blood pressures, and low- and high-density lipoprotein cholesterol approximated normal distributions. Pack-years of smoking were categorized by 10–pack-year intervals. Alcohol intake was categorized as no alcohol or ≤15, 15–30, or >30 g/d. Univariate and multivariate analyses adjusting for age, sex, and vascular risk factors were performed initially. Analyses were then repeated with a gene-alcohol interaction (IL-6 genotype by alcohol category product variable) term included. Binary logistic regression analysis was used to determine odds ratios (ORs) for IL-6 levels in the highest quartile and for the presence of carotid plaque in the case-control study. Quadratic curve estimation was used to test for the presence of a J-shaped relationship between alcohol, IMT, and IL-6 levels.

Results

IMT Study
DNA for genotyping and IMT measurements was available for all 1000 subjects. The IL-6-174 genotype distribution (GG, 32%; GC, 50%; and CC, 19%) was in Hardy Weinberg equilibrium (P = 0.99). There were no differences between genotypes with regard to age, sex, blood pressure, lipids, HbA1c, BMI, pack-years, average alcohol intake, or socioeconomic status.

IL-6-174 Genotype and IL-6 Levels
Age, current smoking, BMI, and diabetes mellitus were all associated with elevated IL-6 levels (in the top quartile). An inverse relationship was seen for high-density lipoprotein cholesterol (Table 1). The relationship between alcohol intake
and IL-6 levels was J-shaped, with nondrinkers and heavy drinkers having the highest IL-6 levels ($P=0.035$ for quadratic trend) (Figure 1A).

Median (interquartile range) IL-6 levels by genotype were as follows (in pg/mL): GG, 1.51 (1.46); GC, 1.56 (1.30); and CC, 1.87 (1.86). Individuals with the CC genotype had significantly higher IL-6 levels compared with those with either the GG or GC genotype; ORs for IL-6 in the top quartile were 1.68 (95% CI, 1.00 to 2.82; $P=0.049$) after age and sex adjustment and 2.07 (95% CI, 1.16 to 3.72; $P=0.014$) after adjustment for confounding variables (Table 1.)

Figure 2A shows the relationship between alcohol consumption and IL-6 levels according to genotype. There was a significant interaction between genotype and alcohol consumption on IL-6 levels ($P=0.014$ for interaction). Specifically, alcohol drinkers with the CC genotype had higher IL-6 levels (for IL-6 in the top quartile: OR, 2.54; 95% CI, 1.27 to 5.08; $P=0.008$) on multivariate analysis. In contrast, no genotype differences in IL-6 levels were evident in nondrinkers (OR, 0.94; 95% CI, 0.36 to 2.40; $P=0.904$).

**IL-6-174 Genotype and Carotid IMT**

The relationship between alcohol consumption and IMT was J-shaped, with moderate drinkers having the lowest IMT values ($P=0.021$ for quadratic trend) (Figure 1B). Mean CCA-IMT measures by genotype were as follows (in mm): GG, 0.77 (0.14); GC, 0.77 (0.14); CC, 0.79 (0.16). Individuals with the CC genotype had significantly higher IMT values ($P=0.045$) after adjustment for age and sex, but this association was no longer significant on multivariate analysis. Elevated IL-6 levels were associated with increased CCA-IMT ($\beta=-0.157$, $P=0.001$) on univariate analysis, but this finding was no longer significant after additional adjustment for vascular risk factors ($\beta=-0.020$, $P=0.692$).

There was a significant interaction ($P=0.009$) between genotype, alcohol, and CCA-IMT (Figure 2B). The J-shaped relationship between alcohol intake and IMT was seen only for the CC genotype ($P<0.001$ for quadratic trend). Drinkers of $>30$ g/d alcohol who were homozygous for the C allele had an elevated IMT compared with either the GG or GC genotypes ($P=0.001$) after adjustment for age and sex (Figure 2B). This association remained ($P=0.004$) after additional controlling for vascular risk factors, including smoking status and pack-years. These findings were significant for both men and women and for IMT measures taken from the left or right sides of either the CCA or the internal carotid artery.

**Carotid Plaque Case-Control Study**

Similar to the findings for IMT, the relationship between alcohol intake and carotid plaque was J-shaped, with moderate drinkers having the lowest risk for carotid plaque.

The distribution of the IL-6-174 polymorphism was not significantly different between the 2 groups (for cases: CC, 16%; GC, 52.1%; GG, 32%; for controls, CC, 19.5%; GC, 48.9%; and GG, 31.5%; $P=0.541$). Table 2 shows the interaction between genotype and alcohol intake. Consistent with the results of the IMT study, among CC genotypes, a J-shaped relationship between alcohol intake and risk of plaque was seen (Table 2). CC homozygotes who drank heavily were at greatest risk for carotid plaque. The OR for

### Table 1. Multivariate ORs for Elevated IL-6 Levels (Top Quartile)

<table>
<thead>
<tr>
<th></th>
<th>OR (95% CI)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.07 (1.01–1.14)</td>
<td>0.024</td>
</tr>
<tr>
<td>Female sex</td>
<td>1.69 (0.96–2.95)</td>
<td>0.067</td>
</tr>
<tr>
<td>Current smoking</td>
<td>6.40 (3.25–12.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI</td>
<td>1.14 (1.07–1.23)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean systolic blood pressure</td>
<td>1.01 (0.99–1.02)</td>
<td>0.416</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>4.82 (1.71–13.6)</td>
<td>0.003</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>1.00 (0.99–1.01)</td>
<td>0.939</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.98 (0.96–1.00)</td>
<td>0.039</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td>0.96 (0.55–1.69)</td>
<td>0.886</td>
</tr>
<tr>
<td>IL-6-174 CC genotype*</td>
<td>2.07 (1.16–3.72)</td>
<td>0.014</td>
</tr>
</tbody>
</table>

*Multivariate binary logistic regression comparing CC with GC/GG combined.

Figure 1. Relationships between alcohol intake and both IL-6 levels (A; $P$ for quadratic trend = 0.035) and mean CCA-IMT (B; $P$ for quadratic trend = 0.021).
The presence of plaque for drinkers of >30 g/d alcohol with the CC genotype compared with GG homozygotes was 3.64 (95% CI, 1.15 to 11.54; \( P \leq 0.028 \)). For CC compared with any carriage of the G allele (GC/GG), the OR was 2.69 (95% CI, 1.03 to 7.07; \( P \leq 0.044 \)). In contrast, the OR for plaque among CC moderate drinkers (15 to 30 g/d) was 0.34 (95% CI, 0.08 to 1.35) Differences between genotypes were not significant at lower levels of alcohol intake.

**Discussion**

This study confirms the previously reported J-shaped relationship between alcohol intake and both carotid IMT and plaque.\(^7\)\(^8\) This is similar to the J-shaped relationship found between alcohol and clinical cardiovascular disease and suggests that this is mediated, at least in part, by atherosclerosis. Our results add to the understanding of this relationship in 2 ways. First, we have demonstrated that genetic predisposition influences the relationship. The J-shaped relationship between alcohol intake and both increased IMT and carotid plaque was seen only for the proinflammatory CC genotype of the IL-6-174 polymorphism. For heavy drinkers (>30 g/d), consistent findings of increased IMT and greater prevalence of carotid plaque were seen in CC homozygotes. Second, our results provide further evidence that inflammation, as determined by serum IL-6 levels, forms part of the causal link between alcohol intake and the increased atherosclerosis seen in heavy drinkers. The IL-6-174 polymorphism may explain some of the excess risk of atherosclerosis seen in heavy drinkers. Differences between genotypes were not significant in nondrinkers; therefore, this polymorphism cannot account for the increased risk seen in nondrinkers.

Increasing evidence suggests that inflammation is important in the pathogenesis of atherosclerosis, stroke, and ischemic heart disease. In prospective studies, elevated basal IL-6 levels are predictive of future vascular events.\(^11\) Although inflammatory markers have been independently associated with increased IMT, they also correlate strongly with conventional cardiovascular risk factors.\(^2\)\(^–\)\(^4\) In this study, a number of risk factors strongly correlated with IL-6 levels and with mean CCA-IMT. IL-6 levels correlated strongly with IMT, but this association was no longer significant when these conventional cardiovascular risk factors were controlled for. This is consistent with conventional cardiovascular risk factors, including alcohol, influencing atherosclerosis risk via inflammation and cytokine responses.

Previous studies have suggested that the IL-6-174 polymorphism is associated with quantitative changes in inflammatory markers. Carriers of the C allele have been found to have both higher baseline C-reactive protein levels\(^13\)\(^,\)\(^23\) and higher peak IL-6 levels after coronary artery bypass surgery.\(^24\) The risk of elevated IMT was confined to CC homozygotes, with no clear heterozygote effect. This suggests that there may be a threshold effect, with only very high levels of IL-6 predisposing to disease. In the case-control study, the prevalence of plaque did show a trend toward a gene-dose effect in heavy drinkers, but again this was only statistically significant for the CC genotype. Previous studies have found associations between the C allele and both myocardial infarction\(^4\)\(^,\)\(^15\) and prospective cardiovascular and all-cause mortality in patients with abdominal aortic aneurysm.\(^16\) We have studied 2 steps in the early pathogenesis of atherosclerosis, and it may be that the heterozygote effect becomes apparent only in more advanced vascular disease.

Cross-sectional studies have shown that increased common carotid IMT is both a marker of atherosclerosis elsewhere in
the arterial system\textsuperscript{25} and an independent predictor of future stroke and myocardial infarction risk.\textsuperscript{26–29} There is evidence for a strong genetic component of carotid IMT, with genes accounting for 30\% to 66\% of IMT variability.\textsuperscript{1} CCA-IMT measures allow subclinical disease to be studied in a population sample and are therefore less prone to bias because of population stratification, which can be a problem with case-control candidate gene studies. A criticism of using IMT as a surrogate measure of atherosclerosis is that lesser degrees of IMT thickening may reflect an adaptive response to stress rather than atherosclerosis per se.\textsuperscript{30} For this reason, in addition to analyzing IMT, we also determined the presence of carotid artery plaque as a marker of established and more advanced atherosclerosis.\textsuperscript{31} In both subpopulations, we found similar relationships between the IL-6 polymorphism, alcohol, and vascular damage, demonstrating consistency of the relationship.

In this study, we used mean CCA-IMT measured with automated software-analysis. A number of methods for measuring IMT have been described in the literature that vary in the arterial sites and number of points measured. Analysis can use either the mean or the maximum IMT, and measurements can be performed either manually or with automated edge-tracking software. A review of these methods has suggested that measurements from the CCA are more reproducible than at other sites and that offline automated edge-tracking methods, similar to the one used in this study, may have advantages over manual measurements.\textsuperscript{31}

The observed interaction between IL-6 genotype and alcohol remained significant after controlling for the major lifestyle determinants of IL-6 levels, including BMI and smoking parameters. This supports the hypothesis that alcohol intake per se rather than associated lifestyle differences mediates this enhanced inflammatory response. In vitro work on the IL-6 promoter region also provides possible biological explanations for the observed interaction. IL-6 expression is regulated by transcription factors located in the promoter region of the gene.\textsuperscript{32–34} Ethanol activates the IL-6 transcription factor nuclear factor kappa B in vitro.\textsuperscript{35} Heavy alcohol consumption also affects glucocorticoid secretion.\textsuperscript{36} Activated glucocorticoid receptor represses IL-6 gene expression by occlusion of the multiple response element enhancer region and basal IL-6 promoter elements, which includes the site of the –174 polymorphism.\textsuperscript{34} The –174 polymorphism, leading to altered binding affinity for these transcription factors, might explain why the CC genotype results in increased IL-6 levels in the face of heavy alcohol consumption.

In summary, our data suggest that the functional IL-6-174 polymorphism is a mediator of increased inflammation and atherosclerosis in heavy alcohol drinkers and support the hypothesis that inflammation may form part of the intermediate causal pathway between heavy alcohol consumption and atherosclerosis.

Acknowledgments

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References


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